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The *in vitro* respiratory toxicity of cristobalite-bearing volcanic ash

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ABSTRACT

Ash from dome-forming volcanoes poses a unique hazard to millions of people worldwide due to an abundance of respirable cristobalite, a crystalline silica polymorph. Crystalline silica is an established respiratory hazard in other mixed dusts, but its toxicity strongly depends on sample provenance. Previous studies suggest that cristobalite-bearing volcanic ash is not as bio-reactive as may be expected for a dust containing crystalline silica. We systematically address the hazard posed by volcanic cristobalite by analysing a range of dome-related ash samples, and interpret the crystalline silica hazard according to the mineralogical nature of volcanic cristobalite. Samples are sourced from five well-characterized dome-forming volcanoes that span a range of magmatic compositions, specifically selecting samples rich in cristobalite (up to 16 wt%). Isolated respirable fractions are used to investigate the *in vitro* response of THP-1 macrophages and A549 type II epithelial cells in cytotoxicity, cellular stress, and pro-inflammatory assays associated with crystalline silica toxicity. Dome-related ash is minimally reactive *in vitro* for a range of source compositions and cristobalite contents. Cristobalite-based toxicity is not evident in the assays employed, supporting the notion that crystalline silica provenance influences reactivity. Macrophages experienced minimal ash-induced cytotoxicity and intracellular reduction of glutathione; however, production of IL-1 β , IL-6 and IL-8 were sample-dependent. Lung epithelial cells experienced moderate apoptosis, sample-dependent reduction of glutathione, and minimal cytokine production. We suggest that protracted interaction between particles and epithelial cells may never arise due to effective clearance by macrophages. However, volcanic ash has the propensity to incite a low, but significant, and sample-dependent response; the effect of this response *in vivo* is unknown and prolonged exposure may yet pose a hazard.

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1. Introduction

Given the established hazard posed by respirable crystalline silica (CS) in occupational settings, the presence of cristobalite, a CS polymorph, in ash emerged as a primary health concern during the 1980 eruption of Mount St. Helens, USA (Green et al., 1981), and again during the 1995-present eruption of Soufrière Hills volcano, Montserrat (Baxter et al., 1999; Wakefield, 2000), gaining the attention of governments and international public health and toxicology experts (Baxter et al., 2014). As a result, detection of CS in volcanic ash has been undertaken in response to many

eruptions worldwide, and is central to rapid hazard assessments carried out by the International Volcanic Health Hazard Network (e.g., Damby et al., 2013; Horwell et al., 2013a). However, the consequence of regular exposure to volcanic cristobalite is still unknown, even in areas with prolonged exposure; for example, the densely populated regions surrounding Mt. Sakurajima, where persistent small eruptions frequently expose the population to ash containing up to 5 wt% cristobalite (reviewed by Hillman et al. (2012)).

Comprehensive risk assessments to determine the long-term risk of silicosis arising from chronic exposure to cristobalite-bearing ash have been conducted only for the eruptions of Mount St. Helens and Soufrière Hills (Horwell and Baxter, 2006). As a non-occupational risk of silicosis had never been recognized prior to the Mount St. Helens eruption, these assessments relied on occupational approaches, for which determination of exposure to

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crystalline silica is central; however, the toxicity of volcanic cristobalite had not yet been established (Baxter et al., 2014). Despite substantial variability in discrete *in vitro* and *in vivo* toxicology results for volcanic ash to date (see Baxter et al. (2014) and Horwell and Baxter (2006) for a full review), there is a tacit assumption in the literature that observed toxicity may result from the presence of CS (e.g., Sanders et al., 1983; Wehner et al., 1986). However, no notable intra-study differences in reactivity among samples with variable cristobalite contents have been observed (e.g., Housley et al., 2002; Vallyathan et al., 1983; Wilson et al., 2000).

The respiratory hazard posed by CS in other mixed dusts is dependent on the provenance of the sample, leading CS reactivity to be deemed a 'variable entity' (Donaldson and Borm, 1998). Volcanic cristobalite is particularly abundant in ash from dome-forming volcanoes (Damby, 2012), where mounds of viscous lava pile up over the vent. The active dome environment facilitates the *in situ* crystallization of cristobalite in these lavas through vapour-phase deposition in cracks and pore space and through devitrification of volcanic glass (Baxter et al., 1999; Horwell et al., 2013b). The presence of cristobalite in ash, therefore, requires incorporation of existing lavas during an eruption, and can comprise upwards of 25 wt% of the ash (Damby, 2012; Horwell et al., 2014). Its abundance depends on dome residence time and lava extrusion rate as well as the eruptive style by which the ash is generated (Horwell et al., 2014). For example, ash from the initial 2008 eruption of Chaitén volcano contained ~2 wt% cristobalite, whereas subsequent eruptions after dome growth began contained 13–19 wt% (Horwell et al., 2010).

The influence of source environment and mineralogy on toxicity has recently been considered for volcanic cristobalite, whereby natural substitutions of aluminium for silicon are shown to affect the chemistry and structure of volcanic cristobalite relative to pure-phase samples (Damby et al., 2014; Horwell et al., 2012). Horwell et al. (2012) suggest that these substitutions could potentially reduce the toxicity of volcanic cristobalite, as aluminium has been shown experimentally to quell CS toxicity (Duffin et al., 2001). Further, Horwell et al. (2012) show that discrete ash grains from Soufrière Hills volcano are mineralogically heterogeneous, effectively reducing the surface area of cristobalite at the particle surface.

Despite the present geological understanding, interpreting and contextualizing the hazard posed by different and/or new volcanic eruptions has been difficult. Direct inter-study comparisons are problematic as variably sourced ash comprises different mineralogical constituents and eruptive histories, and because previous toxicological assessments typically focus on single locations. Only two studies have considered multiple source volcanoes: the first, by Vallyathan et al. (1984), considered three volcanoes (Mount St. Helens, El Chichón, and Galunggung); however, their respirable samples only contained ~1.5 to 2.0% CS; the second, by Horwell et al. (2013a), benchmarks cristobalite-free ash from the 2010 and 2011 eruptions of Eyjafjallajökull and Grímsvötn, Iceland against a

cristobalite-rich sample from Soufrière Hills volcano. Inter-study comparisons are further hindered by differences in experimental design. For example, haemolysis assays on Soufrière Hills ash by Cullen et al. (2002) and Wilson et al. (2000) both use sheep erythrocytes and a top particle dose of 20–30 mg ml⁻¹ whereas Horwell et al. (2013a) use fresh human erythrocytes and a top dose of 1 mg ml⁻¹. Consequently, Soufrière Hills ash has ranged from 0 to 45% haemolysis in studies that cannot be directly compared. Within these studies, samples from the same volcano, and even same eruptive phase, have elicited different responses (e.g., Horwell et al. (2013a) and Wilson et al. (2000)).

Here, we systematically evaluate the *in vitro* response elicited by respirable cristobalite-bearing volcanic ash from five dome-forming volcanoes, specifically selecting samples rich in CS, to understand the toxicity of volcanic cristobalite. This is first study to specifically investigate the reactivity of volcanic cristobalite as well as the first to consider the toxicity of ash derived from an exceptional range of magmatic compositions. We assess the ash-induced decrease in viability and pro-inflammatory activation of macrophages and alveolar type II epithelial cells. Macrophages are classically considered a crucial cell type in the initiation and progression of pulmonary inflammation due to their wide spectrum of pro-inflammatory cytokine production and direct role in particle clearance, and type II epithelial cells are thought to play a key role in pulmonary inflammation and fibrogenesis, including in response to CS exposure (Driscoll et al., 1993). Ash samples from Mount St. Helens and Soufrière Hills volcano (discussed above) contextualize the present study due to the wealth of previous health-focused studies (see Horwell and Baxter (2006) for a review). Further cristobalite-bearing samples are analysed from Mt. Unzen (Japan), Volcán de Colima (Mexico) and Mt. Merapi (Indonesia) due to their location in densely populated areas and history of violent eruptions, factors which led to their designation as Decade Volcanoes by the United Nations as part of a disaster-reduction effort; thus, understanding the respiratory hazard posed by these volcanoes is crucial.

2. Methods

2.1. Samples of volcanic ash

Volcanic ash samples were sourced from 5 dome-forming volcanoes, ranging from basaltic-andesite to dacite in composition (Table 1). Samples were selected from a larger sample suite to maximize the CS contents of the samples analysed (see Damby (2012)). Mineralogically, these samples primarily comprise volcanic glass, feldspars, pyroxenes, amphiboles, Fe–Ti oxides, and crystalline silica polymorphs (see Supplementary material). All samples were generated from eruptions involving dome material but were derived from a range of eruptive styles due to the different eruptive natures of the volcanoes studied. Two samples were selected from Merapi to consider the effect of different

Table 1

Sample information for the respirable ash panel. All ash samples were generated in the presence of a volcanic dome. Cristobalite abundance was determined by the IAS method of Le Blond et al. (2009) and has a ± 3 wt% error.

Sample	Source	Ash type	Eruption date	Collection date	Distance to source (km)	Cristobalite (wt%)
COL	Colima, Mexico	Vulcanian explosion	unknown	21/03/2010	<0.2	5.7
MER	Merapi, Indonesia	Dome collapse	11–19/07/1998	09/08/1998	0.2	2.7
MER.PDC	Merapi, Indonesia	Pyroclastic density current	05/11/2010	30/11/2010	17.0	6.2
MSH	Mount St. Helens, USA	Vent explosion	08/03/2005	10/03/2005	6.5	15.3
SHV ^a	Soufrière Hills, Montserrat	Dome collapse	05/06/1999	05/06/1999	4.0	15.7
UNZ	Unzen, Japan	Dome collapse	03/04/1993	03/04/1993	5.0	7.3

^a SHV is the same as sample MRA5/6/99 in Horwell et al. (2003).

fragmentation mechanisms on the particle hazard and are distinguished by the designation 'PDC', which denotes the sample derived from a pyroclastic density current (PDC) deposit rather than ashfall. Further information on each volcano can be found in the [Supplementary material](#).

Respirable fractions were isolated at SAFENANO, Institute of Occupational Medicine, Edinburgh. Approximately 5 g of bulk volcanic ash was aerosolised with HEPA-filtered compressed air using an AERO PA100 particle aerosoliser. The aerosol was passed through a gravitational separator where large particles and agglomerates sedimented from the aerosol in accordance with Stoke's Law. Particles below a maximum theoretical aerodynamic diameter of 6 μm (i.e., within the respirable range; [Brown et al., 2013](#)), calculated for a specific airflow and particle density in the system, then entered a sampling chamber for collection.

2.2. Particle size distribution and morphologies

The particle size distribution of the respirable particulate aerosols was analysed concurrent with separation by two real-time instruments: a Fast Mobility Particle Sizer (FMPS; model 3091, TSI Inc.) and an Aerodynamic Particle Sizer (APS; model 3320, TSI Inc.). The FMPS measures particles from 5.6 to 560 nm based on electrical mobility and the APS measures particles from 500 to 20,000 nm by light scattering. By overlapping the two instrument readings at their detection boundaries, a total range of 5.6 nm to 20 μm was analysed. Data were recorded over a 15–30 min period.

Size-classified respirable ash was imaged by scanning electron microscopy (SEM) to confirm the upper particle size cut-off and to consider particle morphology and cristobalite habit. Cristobalite was confirmed by energy dispersive X-ray spectroscopy (EDS). Imaging and chemical analysis was carried out on a Hitachi SU-70 FEG SEM equipped with an Oxford Instruments EDS system (IN-CAX-act LN2-free analytical Silicon Drift Detector) in the GJ Russell Microscopy Facility, Department of Physics, Durham University.

2.3. Quantification of crystalline silica phases

The abundance of CS as cristobalite, quartz and tridymite was quantified by X-ray diffraction (XRD) using the IAS method of [Le Blond et al. \(2009\)](#). This method allows for quantification of single phases without prior determination of a sample's bulk mineralogy. Experiments were performed on an Enraf-Nonius X-ray diffractometer with an INEL curved position sensitive detector at the Natural History Museum, London, UK. Single phases were quantified as outlined in [Le Blond et al. \(2009\)](#) using Nonius GUF software. Phase quantifications are an average of 2 experiments and have ± 3 wt% error.

2.4. Treatment of cell cultures with respirable ash

The human leukaemic monocytic THP-1 cell line was maintained in continuous suspension using RPMI 1640 medium containing 10% heat-inactivated FBS (PAA Laboratories, Ltd., UK) supplemented with 2 mM L-Glutamine, penicillin (100 units ml^{-1}) and streptomycin (100 $\mu\text{g ml}^{-1}$). Cells were seeded at a density of 5×10^5 cells ml^{-1} in 12-well plates and differentiated to express a macrophage phenotype with 10 ng ml^{-1} PMA (Sigma-Aldrich, UK) for 48 h. Prior to particle treatment, the media was replaced with serum-free RPMI media (supplemented as above) for 24 h.

A549, a human adenocarcinoma-derived type II alveolar epithelial cell line, was maintained in continuous culture in DMEM growth media with 10% FBS and supplemented with L-Glutamine, penicillin and streptomycin as per the THP-1 cell media. To prepare cells for particle treatment, cells were trypsinized using a

0.25% solution and allowed to re-adhere overnight. Treatment was performed on 1 ml of cells at a density of 5×10^5 cells ml^{-1} in each well of a 12-well plate. Both cell lines were maintained at 37 °C in a 5% CO_2 environment.

Cells were treated for 24 h with four concentrations of volcanic ash and control particles: 5, 10, 25 and 50 mg cm^{-2} . DQ12 quartz was used as a positive control, as CS is of primary concern and DQ12 toxicity is well established in the assays employed and is utilised extensively in previous ash toxicity studies (see [Horwell and Baxter \(2006\)](#)). TiO_2 (rutile) was used as a negative control. Volcanic ash was suspended in the appropriate cell-line media without FBS and sonicated for 10 min to disaggregate the particles. Mass was used as the metric since mass is the current metric for CS and nuisance dusts in the workplace globally.

2.5. Assessment of cytotoxicity

The extent of cytolysis was determined by lactate dehydrogenase (LDH) release. Culture medium was transferred to 1.5 ml microcentrifuge tubes and spun at 12,000g for 10 min to remove any cell debris and remaining particulate. 35 μl of cell-free culture supernatant was transferred in triplicate to a 96 well plate and each well diluted with 70 μl of phosphate buffered solution (PBS). 100 μl of LDH test reagent (Roche Diagnostics, Mannheim, Germany) was added to each well. The absorbance was determined at 490 nm after 30 min using a Synergy HT microplate reader (BioTek Instruments, Inc., VT, USA). The level of LDH release is expressed as a percentage of total LDH release from complete cell lysis by Triton X-100. Data are expressed as the average of 3 experiments.

Cell viability was determined using the alamarBlue[®] cell proliferation assay. Following particle treatment, the supernatant was removed and the cells washed with PBS. 300 μl of PBS and 30 μl of alamarBlue[®] (Invitrogen, Life Technologies, CA, USA) was added to each well. THP-1 cultures were incubated for 1 h and A549 cultures for 30 min at 37 °C in 5% CO_2 . The solution was spun at 12,000g for 5 min, as the particles interfered with the readings, and transferred to a 96 well plate to be read spectrophotometrically at 570 nm. Cell viability is expressed as a percentage of base line viability (no particle treatment) using Triton X-100 as complete cell death. Data are expressed as the average of 3 experiments.

2.6. Determination of apoptosis, necrosis, and cellular stress

The extent of apoptosis, necrosis and endogenous antioxidant production was determined by fluorescence-activated cell sorting (FACS). FITC-conjugated Annexin V staining was used to determine the percentage of cells actively undergoing apoptosis. Propidium iodide (PI) was used to distinguish viable from non-viable (necrotic) cells. Monochlorobimane (MCB) was used to measure intracellular concentrations of glutathione (GSH), the principal intracellular low-molecular-weight thiol.

Supernatants from post-exposure THP-1 and A549 cell cultures were collected and spun at 1500 RPM for 3 min to recover non-adherent cells. Adherent cell monolayers were washed with PBS (without Ca and Mg) $\times 2$ and trypsinized (5 min, 37 °C, 5% CO_2), the trypsin neutralised with culture media, and recovered cells were added to previously collected cells. Collected cells were washed $\times 3$ at 1500 RPM for 3 min and re-suspended in Annexin buffer (Hank's Balanced Salt Solution with 2.5 mM CaCl_2 , filter sterilised). Cells were stained with 5 μl FITC-Annexin V and 1 μl MCB per 195 μl cell suspension and incubated for 10 min. Cells were spun at 1500 RPM for 3 min and re-suspended in 200 μl Annexin buffer. PI (1 μl) was added immediately prior to flow cytometry. Unstained cells and unstained particle-treated cells were

analysed to control for differences in size and granularity resulting from phagocytosis of ash. Untreated stained cells (negative vehicle controls) were analysed, as were a necrotic control (10 mM H_2O_2), an apoptotic control (amine beads at $50 \mu\text{g ml}^{-1}$), and a GSH control (N-ethylmaleimide; NEM).

2.7. Assessment of the pro-inflammatory response

Enzyme-linked immunosorbent assays (ELISA) were used for the quantification of acute-phase cytokines IL-1 β , IL-6 and chemokine IL-8 produced following particle treatment using cytokine-specific ELISA DuoSet kits (R&D Systems, Abingdon, UK) according to the manufacturer's protocol. Cytokine concentrations were determined spectrophotometrically at 450 nm via extrapolation of a recombinant cytokine protein standard curve.

2.8. Imaging of cell-particle interaction

Differentiated THP-1 cells were imaged by light microscopy following ash treatment to determine whether particles were being internalised. Only THP-1 cells were imaged as A549 aggregated during preparation. Cytospin preparations were made by centrifuging ~ 40 to 60,000 cells in 300 μl of 0.1% BSA/sterile saline solution at 300 RPM for 3 min at room temperature using a Cytospin 4 centrifuge (Thermo Scientific, UK). Air-dried slides were fixed in 100% methanol and stained with eosin (Quick-Diff, Dade Behring, Inc.) and haematoxylin (REASTAIN Quick-Diff Blue), and then submerged in xylene for 30 s. DPX mounting media (Sigma-Aldrich, Poole, UK) was added directly to the cell population and slides were covered with a glass cover slip. Images were captured at 100 \times magnification using QCapture Pro software (Media Cybernetics, Inc., Bethesda, MD).

2.9. Statistical analysis

Statistical analysis was performed using GraphPad PRISM software (San Diego, CA). Multiple comparisons were conducted using two-way analysis of variance (ANOVA) with Tukey's post-test where appropriate. In all cases, tests were considered significant when $p \leq 0.05$.

3. Results

3.1. Particle characterisation

Sizing of the size-separated samples yielded distributions ranging from $\sim 0.08 \mu\text{m}$ to $7.0 \mu\text{m}$ (Fig. 1); the distribution was similar for all samples. A bi-modal distribution arises from combining results from FMPS and APS (Fig. 1); however, this is likely due to instrumentation and is not considered further. The gradual slope towards the upper size cut-off ($\sim 7.0 \mu\text{m}$) is likely a physical artefact as the probability of re-suspension decreases towards the defined particle cut-off ($6 \mu\text{m}$). The lower cut-off ($\sim 0.08 \mu\text{m}$) is not likely representative of a true minimum particle size as particles are subject to aggregation.

SEM imaging of the isolated ash substantiates the particle size data, and reveals little difference in general morphology among samples (Fig. 2a and b). Particles are poorly vesiculated, sub-angular and blocky with varying amounts of nano-scale particles adhering to the surfaces of larger particles. Only scarce mineral fibres were observed in any of the samples. Cristobalite was present in all respirable samples, ranging from ~ 3 to 16 wt% (Table 1). Minor quartz was detected in samples SHV, MSH and UNZ. No tridymite was observed in any sample. SEM backscattered imaging of ash in cross section confirms that discrete ash grains can

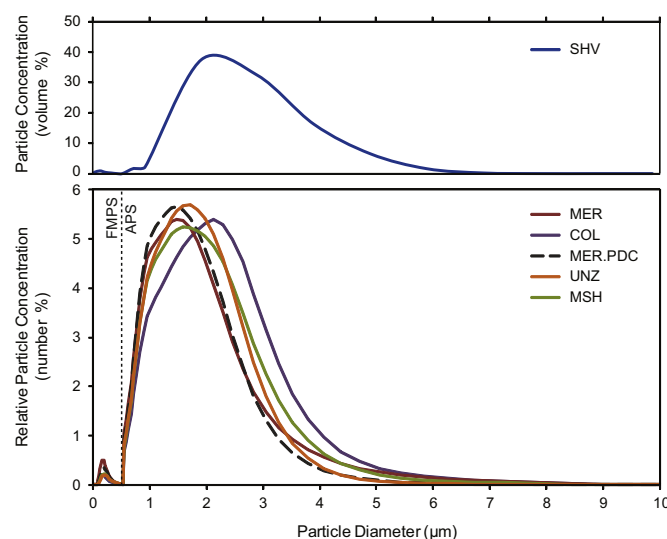


Fig. 1. Particle size distributions for isolated respirable fractions. Size data for particles smaller than $0.5 \mu\text{m}$ are collected by FMPS and data above by APS. Sample SHV was not isolated specifically for this study and therefore does not have equivalent data; however, PSD data from Horwell et al. (2003) are included for comparison.

contain cristobalite intimately associated with other mineral phases and glass (Fig. 2c) whereas others solely comprise cristobalite (Fig. 2d). Further, all cristobalite contains aluminium (Fig. 2d), extending the observations of Horwell et al. (2012) to the respirable fraction.

3.2. Cytotoxicity of differentiated THP-1 and A549 cells

All samples induced some degree of decreased viability in THP-1 macrophages at 24 hours as measured by metabolism of the alamarBlue[®] reagent (Fig. 3a). However, the response to ash did not follow a typical dose-dependent decrease as exemplified by DQ12 quartz. The decrease in viability is similar to that elicited by TiO_2 (consistent 85% viability over the same dose range). A similar response was seen for LDH release (Fig. 3b), where treatment resulted in substantially lower release than DQ12 and marginally lower release than TiO_2 .

A549 cell viability determined by the alamarBlue[®] assay showed little effect of particle treatment compared to DQ12 and TiO_2 (Fig. 3c). Ash samples MER, COL, UNZ elicited more LDH release than TiO_2 but less than DQ12 (Fig. 3d).

3.3. Assessment of apoptosis and necrosis

Differentiated THP-1 cells experienced limited reduction in viability following particle treatment as determined by FACS analysis (Fig. 4); THP-1 cell necrosis and apoptosis were roughly equivalent and negligible. A549 cells experienced a $\sim 20\%$ reduction in viability, which was largely attributed to apoptosis (Fig. 4); all samples resulted in a significant level of apoptosis over untreated control.

3.4. Intracellular oxidative stress

No reduction in GSH abundance was observed with ash treatment in THP-1 cells compared to untreated cells (Fig. 4). However, an ash-induced reduction was observed for A549 cells (Fig. 4), with UNZ and COL causing the most pronounced depletion relative to controls. The extent of the decrease was more pronounced for higher doses; in general, the effect was more prominent than that caused by DQ12 quartz but substantially less than that produced

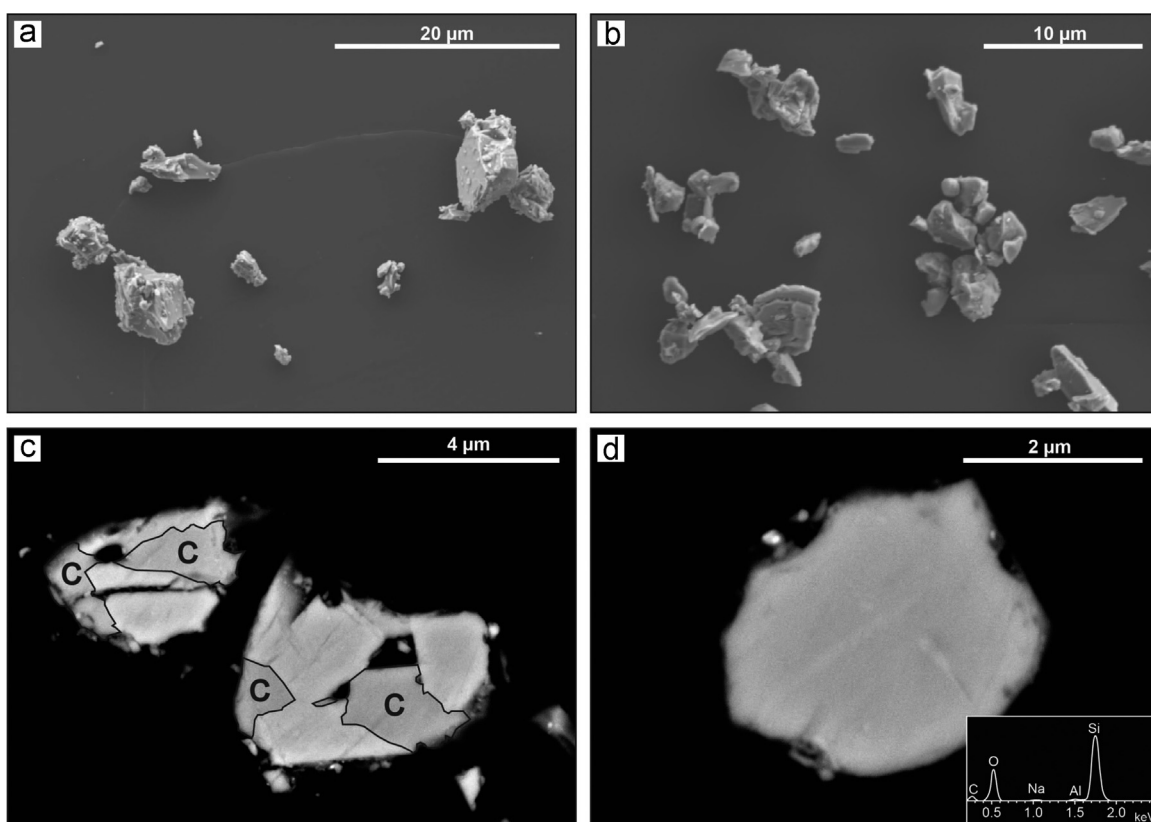


Fig. 2. Scanning electron micrographs of respirable volcanic ash. Selected SEM micrographs of respirable volcanic ash samples (a) UNZ and (b) SHV, which are representative of all samples investigated. Backscattered SEM images of ash sample SHV in cross section showing (c) heterogeneous patches of cristobalite labelled 'c' (cristobalite crystal boundaries are demarcated), and (d) a particle comprised entirely of cristobalite. Inset: EDS spot analysis of cristobalite showing the presence of aluminium and sodium. All images were collected at 6.0 kV and 14 mm working distance.

by NEM, an effective oxidiser of GSH which rapidly depletes it from the cell cytosol.

3.5. Pro-inflammatory response

The pro-inflammatory response of differentiated THP-1 cells was sample and cytokine dependent. The top two doses (25 and $50 \mu\text{g cm}^{-2}$) of SHV, MER and MER.PDC resulted in the production of IL-1 β above the untreated control and were equivalent to the lowest dose ($5 \mu\text{g cm}^{-2}$) of DQ12 (Fig. 5a). Treatment by all samples except for MER resulted in IL-6 production comparable to the untreated control (Fig. 5b). One repeat experiment (of $n=3$) for MER resulted in similar levels to all other samples, resulting in large error bars and perhaps emphasizing the heterogeneous mineralogy of the ash samples. Cells treated with both UNZ and MER produced significantly more IL-8 than untreated cells in a dose-dependent response (Fig. 5c). Levels for the top dose ($50 \mu\text{g cm}^{-2}$) of these samples were also greater than that induced by equivalent concentrations of DQ12. Although MER induced significant IL-8 production, results for MER.PDC were largely negative.

The response of A549 epithelial cells to insult was largely negative (Fig. 5d–f). Little to no release of IL-1 β or IL-6 occurred, especially compared to that induced by DQ12. Production of IL-8 for all particle treatments (ash and DQ12) did not differ significantly from an untreated control ($p > 0.05$), suggesting minimal treatment-dependent production of IL-8 over a basal level.

3.6. Particle-Cell imaging

Light microscope imaging of untreated and ash-treated differentiated THP-1 cells in cytospin preparations shows that particles

were internalised by cells (Fig. 6). Using the techniques here, it was not possible to determine if there was a preferential uptake or retention of certain mineral phases relative to others.

4. Discussion

4.1. Differentiated THP-1 cell response

Phagocytosis of particles by THP-1 macrophages resulted in minimal cytotoxicity, as evidenced by low levels of LDH release, minimal suppression of cellular metabolism, and negligible apoptosis and necrosis. These results agree with previous studies on alveolar macrophages, which generally show no ash-dependent changes in viability (e.g., Fruchter et al., 1980; Green et al., 1982; Huang et al., 2004; Martin et al., 1984); however, one study has shown moderate cytotoxicity to macrophages exposed to Mount St. Helens ash (McLemore et al., 1984). THP-1 cells also appear to have increased intracellular GSH levels following ash exposure. Macrophages are known to have lower levels of intracellular GSH than other cell types (e.g., 10 nmol mg^{-1} protein compared to 150 nmol mg^{-1} protein in A549 cells) (Biswas and Rahman, 2009), and perhaps increase production to cope with insult. This would have the effect of increasing antioxidant defence in response to exposure. Collectively, these cytotoxicity and cellular stress data suggest that clearance by macrophages should be effective with little impairment to the cell at plausible exposures.

It is not possible to assign the pro-inflammatory response of THP-1 macrophages to CS abundance alone. Production of IL-1 β , which is important in the development of silica-related disease (Mossman and Glenn, 2013), was negligible for MSH (as also

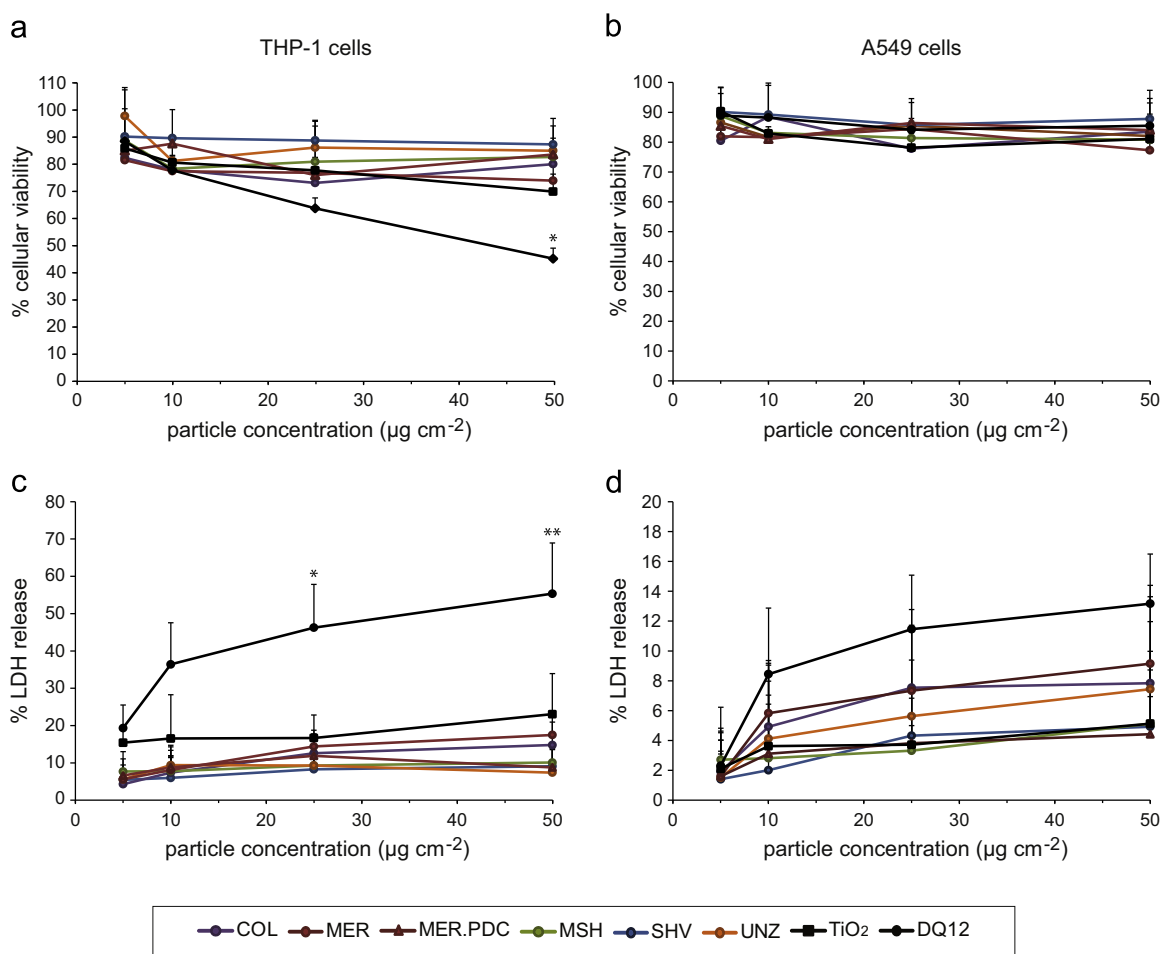


Fig. 3. Cytotoxicity of ash samples towards differentiated THP-1 and A549 cells. Cytotoxicity of THP-1 cells following exposure to respirable ash and controls measured by (a) metabolism of the alamarBlue reagent and (c) LDH release. Cytotoxicity of A549 cells measured by (b) metabolism of the alamarBlue reagent and (d) LDH release. Error bars are the standard error of the mean. * $p \leq 0.05$, ** $p \leq 0.01$.

reported for Mount St. Helens ash by Bonner et al. (1998) and Huang et al. (2004)), which contains the most CS (together with SHV, within error), yet pronounced for MER, which contains the least CS. These data support the adherence of volcanic cristobalite to the 'variable entity' description of CS (Donaldson and Borm, 1998), as proposed by Horwell et al. (2012), whereby 'inherent characteristics and external factors' may affect the propensity of CS to incite IL-1 β production.

An increase in the production of IL-8, a potent chemoattractant and activator of neutrophils, was only observed for low CS-content samples UNZ and MER; a similar inability to stimulate neutrophil chemotactic activity was previously observed for Mount St. Helens ash (Martin et al., 1984). IL-8 transcription is under the control of Nuclear Factor- κ B (NF- κ B), a transcriptional regulator protein complex that becomes activated following CS exposure (Schins and Donaldson, 2000); NF- κ B has been shown to play a role in discriminating between inflammogenic and non-inflammogenic quartz varieties (Duffin et al., 2001). However, like IL-1 β production, IL-8 production does not correlate with CS content. Since NF- κ B is an oxidative stress-responsive transcription factor involved in the pro-inflammatory effects of many pathogenic particles (Schins and Donaldson, 2000), again, CS cannot be independently implicated.

4.2. A549 cell response

In general, A549 cells experienced a moderate reduction in cell viability in response to ash treatment. These data agree with the

results of Wilson et al. (2000), where exposure of A549 cells to three samples of respirable ash from Soufrière Hills resulted in a similar decrease in viability to both DQ12 and TiO₂, which were not statistically different in their study. Results for individual ash samples, however, did not always correspond among assays. For example, COL was the most cytotoxic by LDH and alamarBlue® assays but induced the lowest amount of apoptosis and necrosis. The propensity of all samples towards apoptosis rather than necrosis aligns with previous work using Mount St. Helens ash on lung epithelial cells (Agopyan et al., 2004), and may help explain low haemolysis results for ash in previous studies (e.g., Damby et al., 2013; Horwell et al., 2013a) as membranolysis would constitute necrosis.

Despite the decrease in viability, no pro-inflammatory response by A549 cells was observed above untreated control. Exposure of A549 cells to pure-phase crystalline and amorphous silica is expected to result in the release of IL-8 and IL-6, with CS being the most potent inducer of IL-8 and amorphous silica of IL-6 (Hetland et al., 2001; Monteiller et al., 2007). Contrary to the above studies, the amorphous component of volcanic ash failed to incite production of IL-6, and the CS component of volcanic ash did not result in the production of IL-8 above untreated control, especially considering that both MSH and SHV comprised ~15 wt% cristobalite. However, variability in the response of cultured cells to exposure is well known, and the response can vary over time (Poland et al., 2014).

Lung lining fluid contains high concentrations of antioxidants

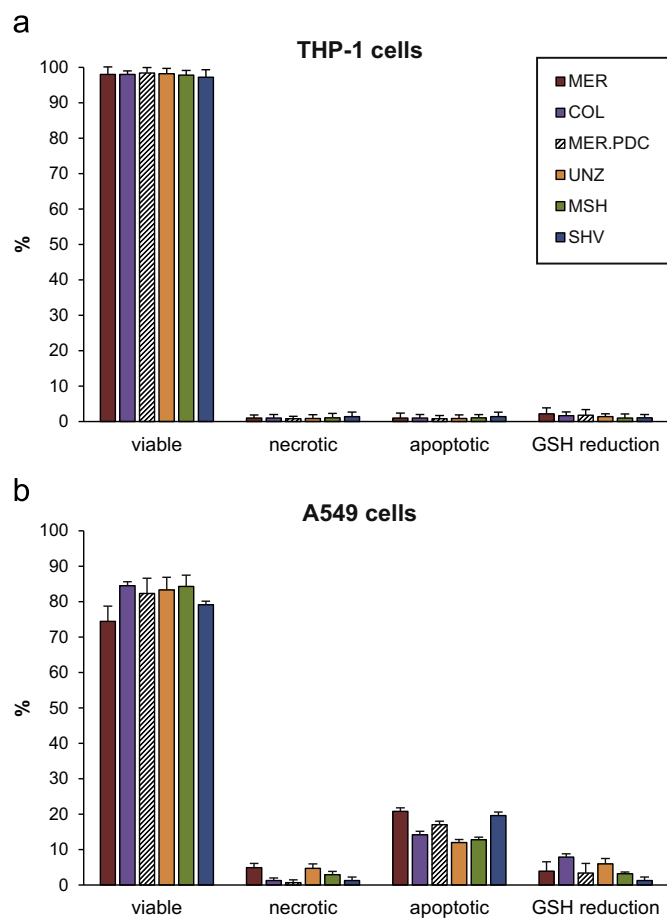


Fig. 4. Viability and intracellular reduction of glutathione (GSH) of THP-1 and A549 cells by flow cytometry. Data are for treatment with $50 \mu\text{g cm}^{-2}$ of volcanic ash and are reported as a per cent of an untreated control, representative of 100% viable ($n=2$). Data are presented in order of increasing cristobalite content.

that form a protective barrier for the lung from oxidative and other damage; depletion of this antioxidant defence can result in oxidative stress to the underlying epithelial cells (Kelly et al., 1996). Depletion of the high concentrations of GSH (a primary antioxidant secreted by epithelial cells) in the lung lining fluid has been observed for various particles and fibres (Brown et al., 2000); however, studies on volcanic ash from Merapi (Damby et al., 2013), and Eyjafjallajökull and Grímsvötn, Iceland (Horwell et al., 2013a) have shown no evidence of particle oxidative capacity in composite respiratory tract lining fluid (including GSH) or ascorbate depletion assays, despite volcanic ash showing pronounced iron-mediated free radical activity (Horwell et al., 2007). Therefore, the extent of intracellular GSH reduction was unexpected. A549 cells are known to have a higher level of GSH than primary epithelial cells (150 nmol mg^{-1} protein versus 60 nmol mg^{-1} protein) (Biswas and Rahman, 2009); therefore, the ability of cells to buffer these effects *in vivo* could be more pronounced.

4.3. Reactivity of volcanic cristobalite

The CS hazard was not evident in cytotoxicity assays nor indicators typically associated with CS toxicity since samples MER and SHV were marginally more reactive than the other samples for all indicators of toxicity (except glutathione reduction in A549 cells) yet contained the least and most cristobalite of all samples analysed ($\sim 3 \text{ wt\%}$ versus 16 wt\% , respectively). Further, no correlation was seen between cristobalite abundance and the toxicity responses evaluated when considering the samples collectively as

a continuum of cristobalite abundances (Table 2). These results agree with the comparative study of respirable ash by Vallyathan et al. (1984), where samples showed wide differences in haemolysis and cytotoxicity despite all samples containing $< 2.0 \text{ wt\%}$ CS. As such, much of the observed toxicity and surface reactivity of ash (in this study and others) stems from factors that cannot be directly attributed to CS content.

These results also complement recent *in vitro* assessments of chronic toxicity using type 1-like (TT1) lung epithelial cells. Analysis with samples from Merapi (Damby et al., 2013), Soufrière Hills (Horwell et al., 2013a), and the Icelandic volcanoes Eyjafjallajökull and Grímsvötn (Horwell et al., 2013a) indicate prolonged production of IL-6, IL-8 and MCP-1; however, as in the present study, the response is not mediated by CS, since the pro-inflammatory response to samples from CS-bearing Merapi and Soufrière Hills (including the bulk sample that corresponds to SHV in the present study) resolved, whereas the response to the CS-free Icelandic samples persisted, and increased in some cases.

The limited apparent CS-based toxicity of cristobalite-bearing ash could plausibly result from two possibilities. First, in adherence to the variable-toxicity theory for CS, the limited reactivity of volcanic cristobalite may reflect compositional or structural modification from pure SiO_2 (due to the substitution of Al), its presence in heterogeneous particles, and/or dissolution of other mineral phases present in volcanic ash (Damby et al., 2014; Horwell et al., 2012). The proportion and presence of other mineral phases will differ due to differences in the source composition and eruptive history of these samples, but the major constituents other than CS were not characterised in detail as these phases have no history of inducing inflammation (Bérubé et al., 2004; Housley et al., 2002); however, they may modulate the response to volcanic cristobalite.

The second possibility is simply that the CS burden per unit dose is not sufficiently high to elicit a response. It is possible that a toxicity threshold exists for the cristobalite content of ash, as observed for CS in other mixed dusts (e.g., 10–15 wt% quartz in coal dust; Le Bouffant et al., 1982), which may be substantiated here by the equivalent production of IL-1 β by THP-1 cells for the top dose of some samples (MER, MER.PDC, and SHV) to the lowest dose of DQ12 (Fig. 5a); however, the practical implications of a higher threshold may be limited since a maximum of 23 wt% cristobalite was observed in a study specifically tracking cristobalite abundance in ash from Soufrière Hills (Horwell et al., 2014). Moreover, as mentioned above, Horwell et al. (2012) show that ash surfaces are heterogeneous (which is confirmed in the present study for respirable ash; Fig. 2c), reducing the available CS. However, this restraint on surface CS alone may be insufficient to account for the limited response, since Jones and Bérubé (2011) implicate a bio-available cristobalite fraction in ash.

4.4. Respiratory hazard of volcanic ash

Exposure to volcanic ash showed little cytotoxicity to macrophages by the assays employed here, limiting concern over the consequences of persistent *in vivo* exposure due to concomitant release of phagocytosed particles from cell death. Therefore, little protracted interaction between the particles and epithelial cells is expected. However, exogenous IL-1 β has been shown to augment alveolar epithelial cell survival by reducing apoptosis (Geiser et al., 2000), and IL-1 β can be provided (to some extent) by macrophages challenged with volcanic ash. *In vivo*, this may facilitate epithelial survival during clearance of ash, but it may also allow cells with DNA damage that should have undergone apoptosis to persist and can be seen as a pre-carcinogenic effect. Indeed, such effects must be considered alongside previously observed type II pneumocyte hyperplasia (Akematsu et al., 1982). These

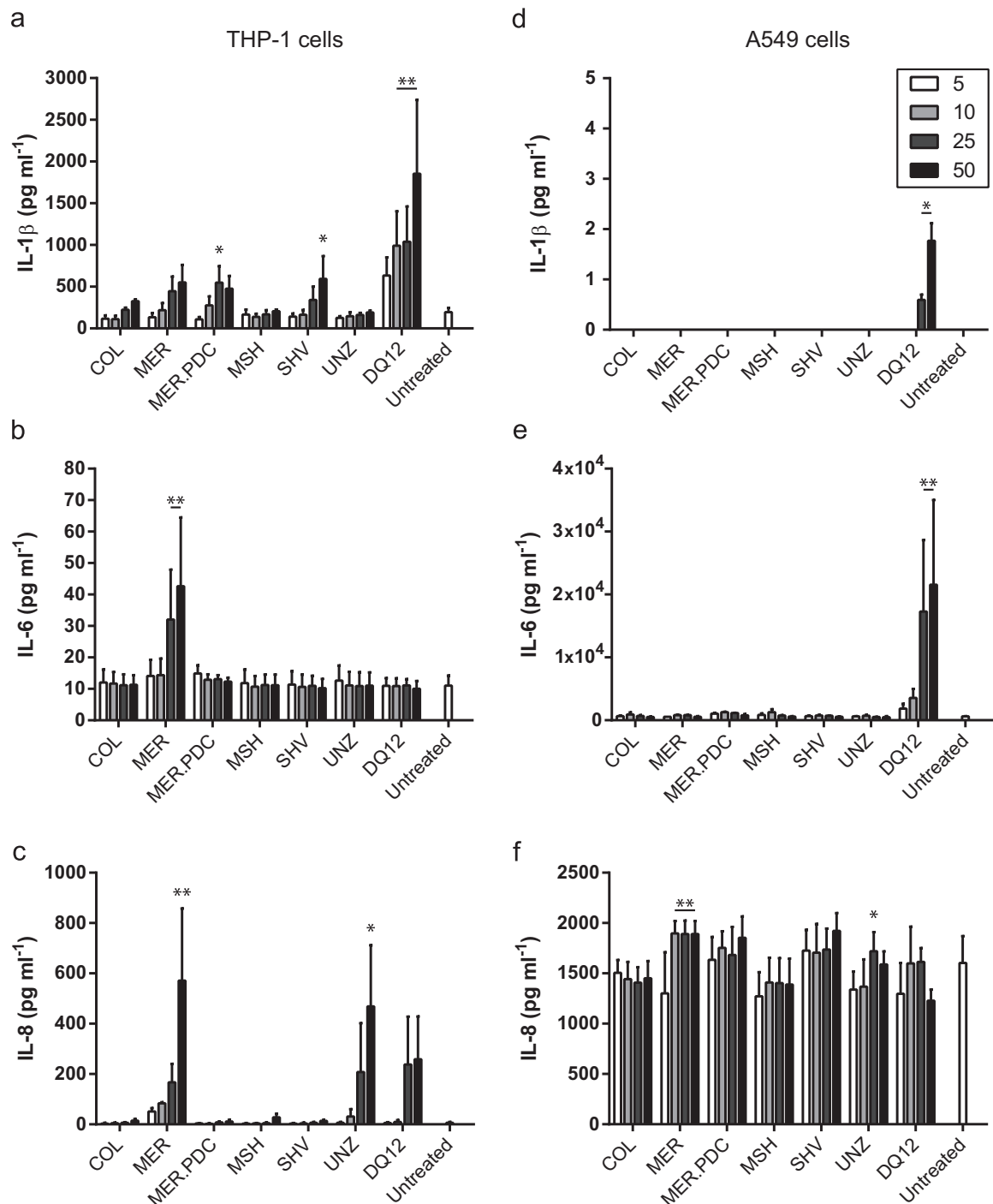


Fig. 5. Cytokine production by THP-1 and A549 cells. Production of pro-inflammatory markers IL-1 β , IL-6 and IL-8 by differentiated THP-1 (a–c) and A549 (d–f) cells at 24 h after treatment with 5, 10, 25 and 50 $\mu\text{g cm}^{-2}$ volcanic ash. Error bars are the standard error of the mean. * $p \leq 0.05$, ** $p \leq 0.01$.

expectations may extend to volcanic ash in general, as *in vitro* exposure to cristobalite-free Eyjafjallajökull ash had minimal effect on innate immunity (Monick et al., 2013), comprising non-statistical impairment of alveolar macrophage function and no decrease in airway epithelial cell integrity.

Previous studies have shown that exposure to both CS and non-CS bearing ash correlates with an increased risk of bacterial infection (e.g., Castranova et al., 1982; Monick et al., 2013), which may be augmented by acute inflammatory effects induced by particle exposure (Sandberg et al., 2012). Opportunistic bacterial infections, like tuberculosis, are widespread in many countries with active volcanism, especially Indonesia, which ranks fifth in

the world for pulmonary tuberculosis (World Health Organisation, 2011), and may be exacerbated by crystalline silica exposure (Balmes, 1990). Evidence from the present study, and those discussed above, indicates a general potential for ash to induce (or exacerbate) inflammatory disorders, irrespective of CS content; as such, we suggest further consideration of complications resulting from ash exposure.

5. Conclusion

The overall effects of a range of cristobalite-bearing ash were low and did not correlate to cristobalite abundance. It is likely a

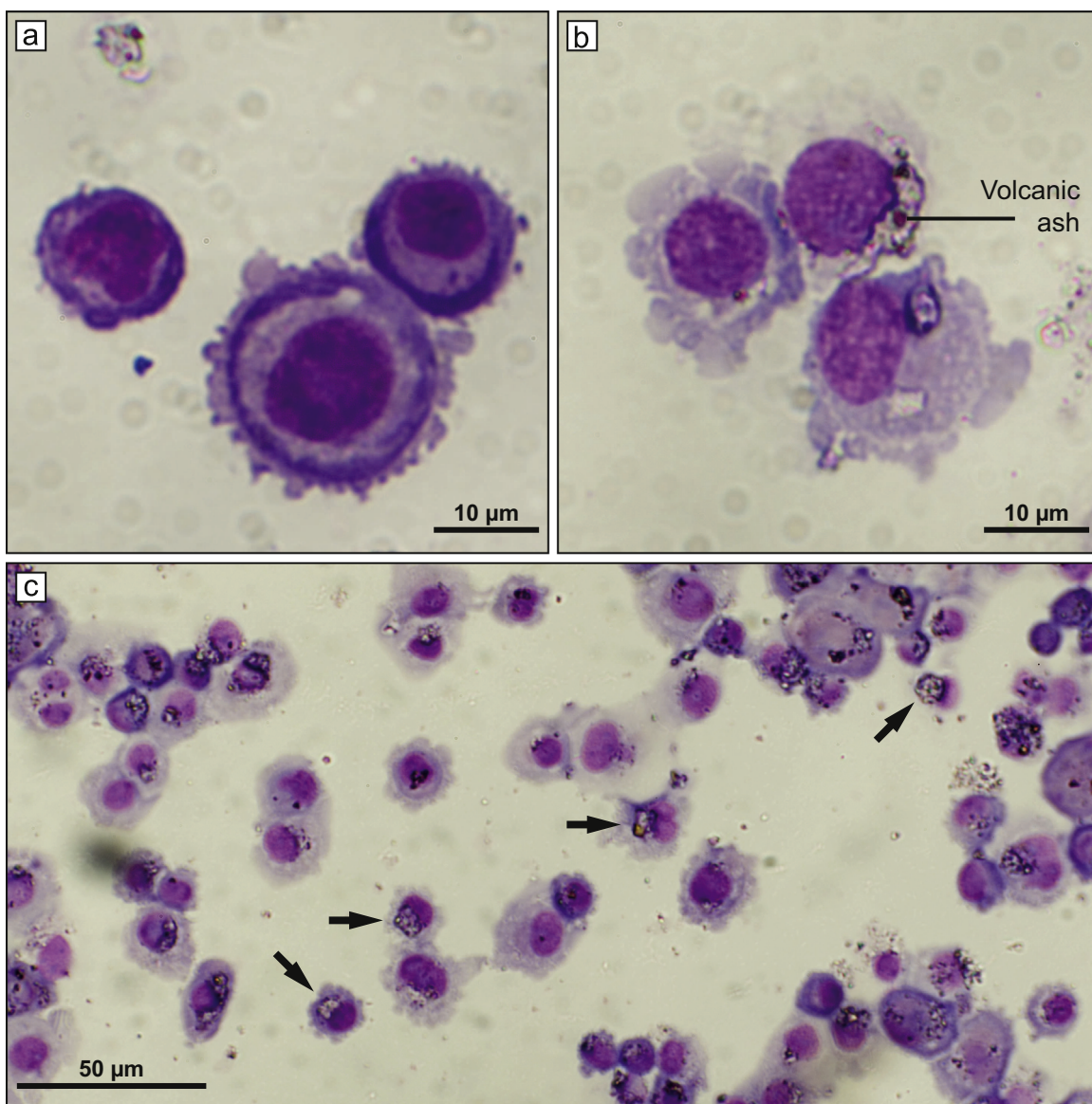


Fig. 6. Uptake of ash by THP-1 cells. Light micrographs of the uptake of volcanic ash by THP-1 cells: (a) untreated cells, (b) cells treated with COL, and (c) low magnification view of cells with internalized ash particles (arrows). Images are representative of all samples.

Table 2

Summary of the correlation (slope and coefficient of determination) between toxicity endpoints and cristobalite content for all samples used in the present study. Individual plots comparing CS to discrete endpoints available in the [Supplementary material](#).

Indicator of toxicity	THP-1 cells		A549 cells	
	Slope	R ² value	Slope	R ² value
Cytokine/chemokine production				
IL-1 β	–	0.0194	n/a	n/a
IL-6	–	0.3387	–	0.0059
IL-8	–	0.3206	–	0.0301
Cellular viability				
FACS	–	0.5347	+	0.0854
LDH release	–	0.0058	–	0.4871
AlamarBlue	+	0.5698	+	0.2900
Oxidative capacity	n/a	n/a	–	0.3373

combination of both ‘intrinsic and external factors’ and dosimetry that accounts for the suppressed reactivity of volcanic cristobalite. However, these data, in conjunction with other studies, indicate the potential for volcanic ash to initiate an inflammatory response without seriously compromising lung cell function or integrity. Consequently, prolonged exposure may yet pose a hazard, and the general potential of ash to incite a response should be considered in risk assessments of long-term complications arising from chronic exposure.

Critically, the results of the present study may not be fully representative of the overall respiratory hazard at the locations studied; as previous reports have emphasized, volcanic ash is a heterogeneous dust, the physicochemical components of which can vary considerably, even during a discrete eruption (e.g., [Horwell et al., 2013a](#)). The influence of this variability is further evidenced here by the disparity in response between explosive and PDC samples. Therefore, volcanic ash should continue to be considered on a case-by-case basis to best inform the hazard posed and most effectively aid mitigation work of disaster managers globally.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at <http://dx.doi.org/10.1016/j.envres.2015.11.020>.

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